Chromatographic Methods: Basics, Advanced HPLC Methods

Chromatography: Basics

Chromatography a physical method for the separation of mixture based on the concept of partition coefficient

Chromatography involves two phases
Mobile phase: a liquid/ gas which caries the mixture to be separated
Stationary phase: through which the mixture is carried by mobile Phase, it can be solid/ liquid

Separations are carried out based on differences in physical and Chemical properties of constituents of a mixture such as
size, shape, mass, charge, boiling point, polarity or chemical affinity

Chromatography: Terminology

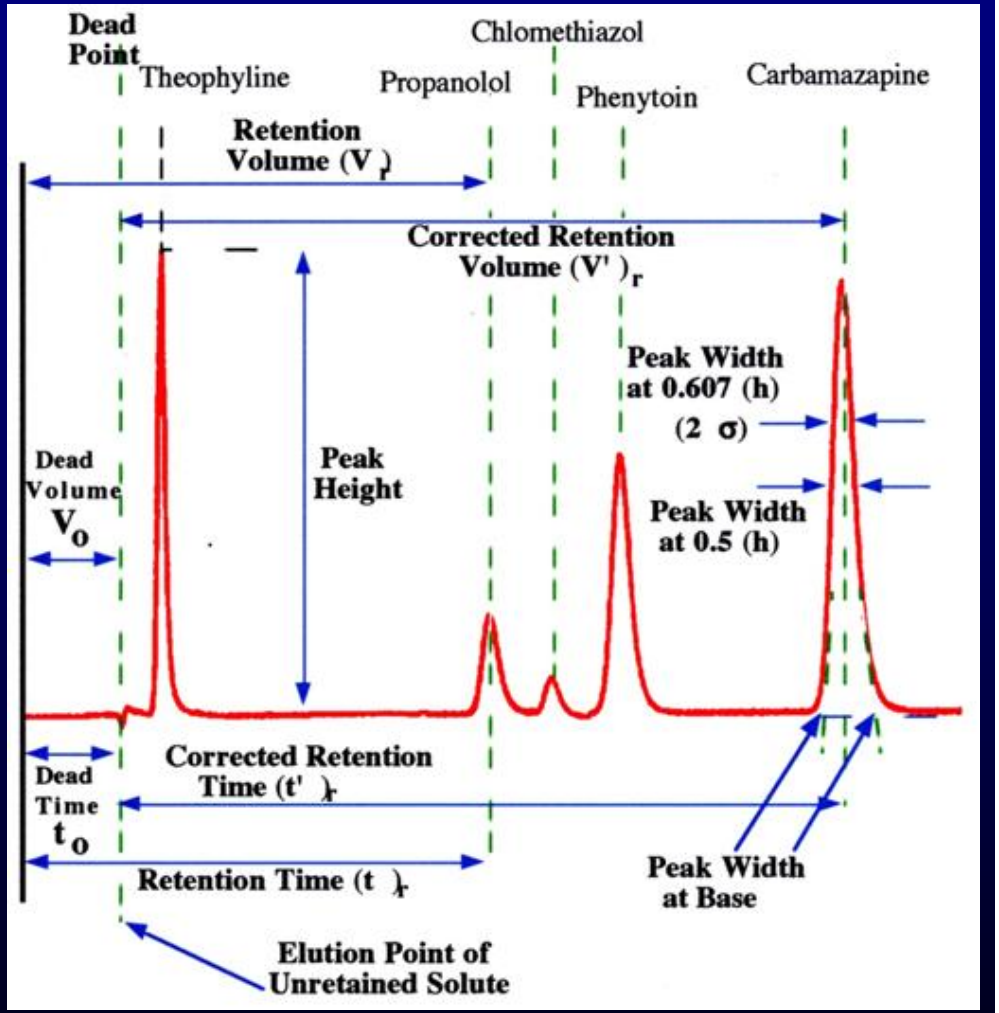
Chromatogram: The visual output of the chromatograph

Retention time: The characteristic time a particular analyte takes to pass through the system i.e. from column inlet to the peak maxima

Retention factor: migration rate of an Analyte on a column

Peak height: The distance between the peak maximum and the base line

Peak width: The distance between each side of a peak measured at certain height of the peak



Chromatography: principle/Theory

the solutes will elute in order of their increasing **distribution coefficients** with respect to the stationary phase

Plate theory: column is considered to be divided into a number of plates

$$N = 5.55^{*} t_R^2 / w_{½}^2$$

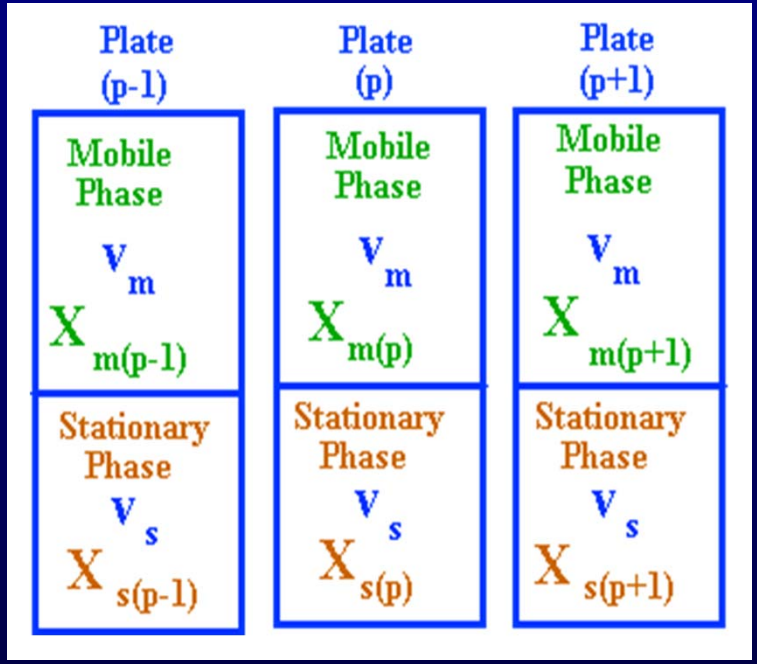
equilibrium must exist in each plate

$$X_s = KX_m$$

(X_m) ; concentration of solute in the mobile phase
(X_s) ; concentration of solute in the stationary phase
(K); distribution coefficient of the solute between the two phases with reference to the stationary phase

$$K = X_s/X_m$$

Chromatography: principle/Theory



chromatography-online.org

the change of mass of solute (dm) in plate (p) will be

$$d_m = (X_{m(p-1)} - X_{m(p)})dV$$

At equilibrium $$d_m = v_s dX_{s(p)} + v_m dX_{m(p)}$$

Chromatography: principle/Theory

$$X_{m(n)} = X_0 \cdot e^{-v} v^n / n !$$

Basic elution curve equation it shows that if (n= no. of theoretical plates) is large, the function tends to the Gaussian function.

$$V_r = V_m + KV_S$$

The **retention volume** depends solely on the distribution coefficient and the volumes of the two phases that are present in the column.

$$K_{(A)} <> K_{(B)} \quad \text{or} \quad V_{S(A)} <> V_{S(B)}$$

The separation of two solutes depends exclusively on the magnitude of their distribution coefficients ($K_{(A)}$) and ($K_{(B)}$) and the amount of stationary phase available to them, ($V_{(A)}$) and ($V_{(B)}$).

Chromatography

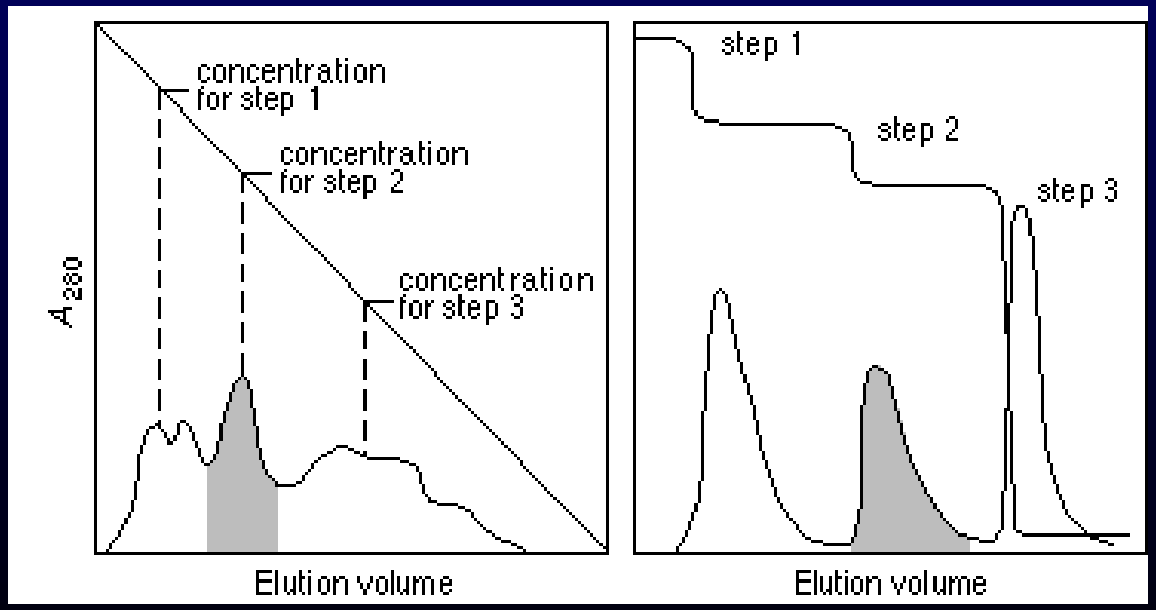
Elution mode

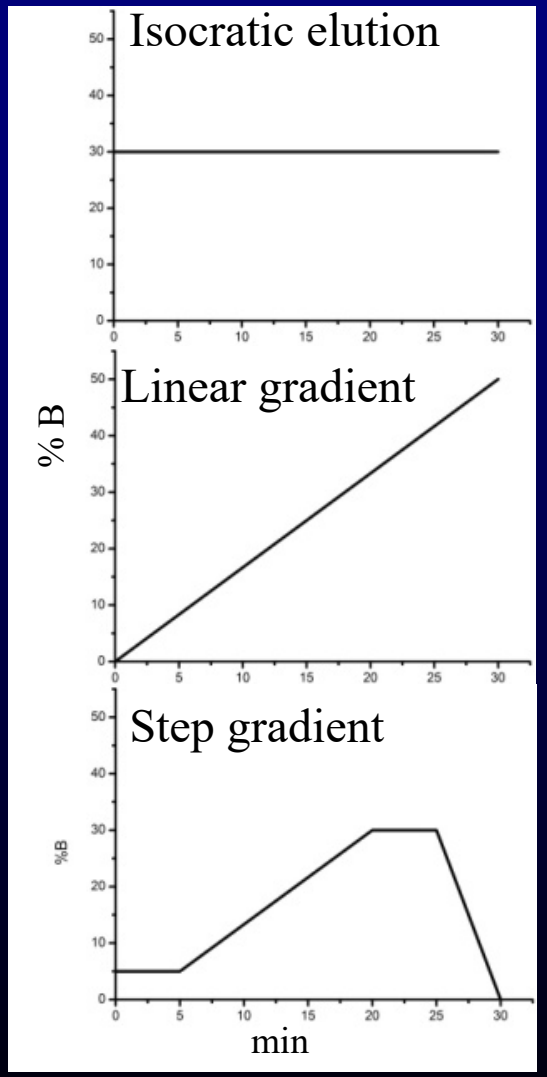
Isocratic elution :

The composition of the mobile phase kept constant through out elution

Gradient elution :

The composition of the mobile phase varied during elution





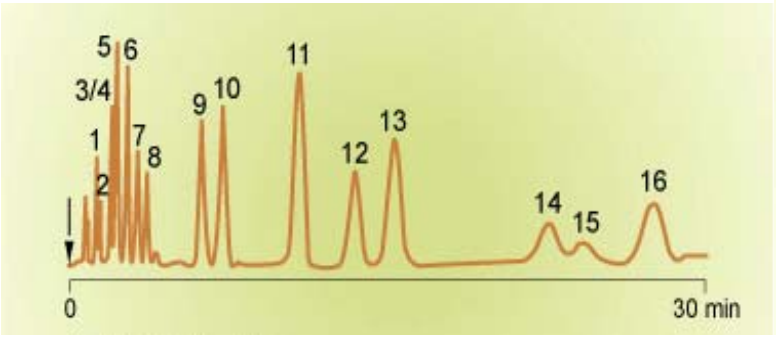
Chromatography

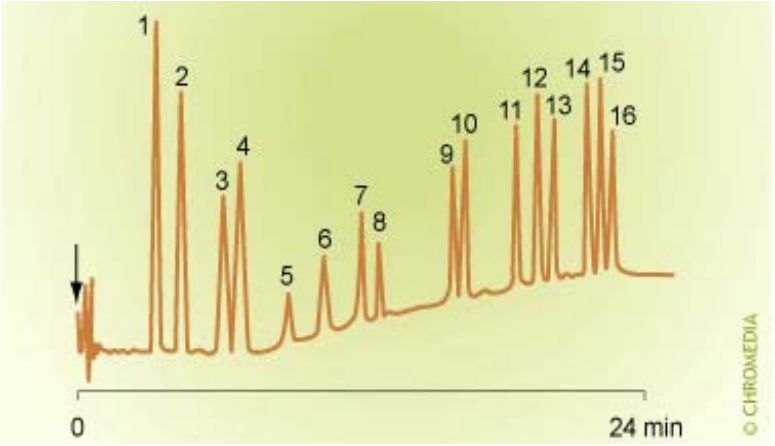
Elution mode

PAH analysis through HPLC

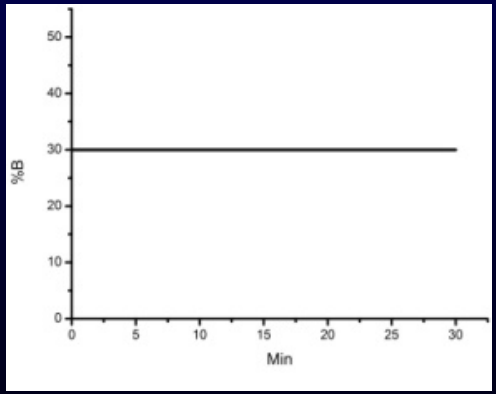
Gradient elution

Isocratic elution

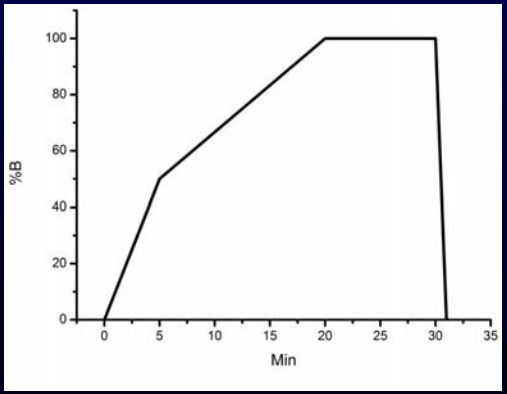




ACN/H_2O 70/30



ACN/H2O
0-5 50/50
5-20 100/0
20-30 100/0



From chromedia.org

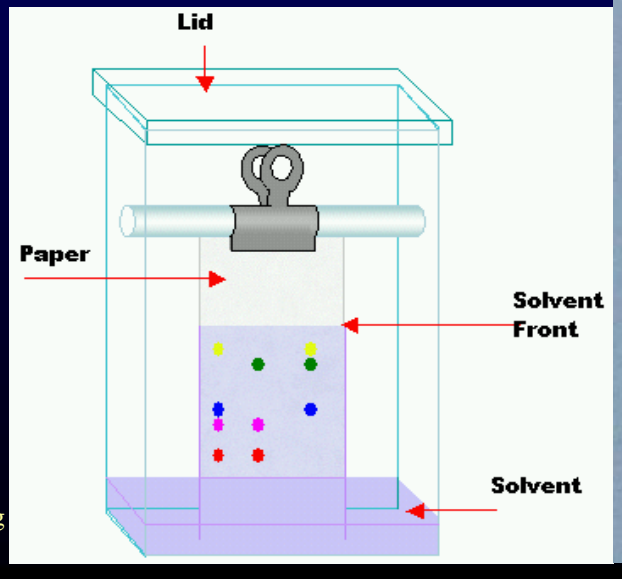
Chromatography: Types

Based on shape of chromatography (stationary phase)

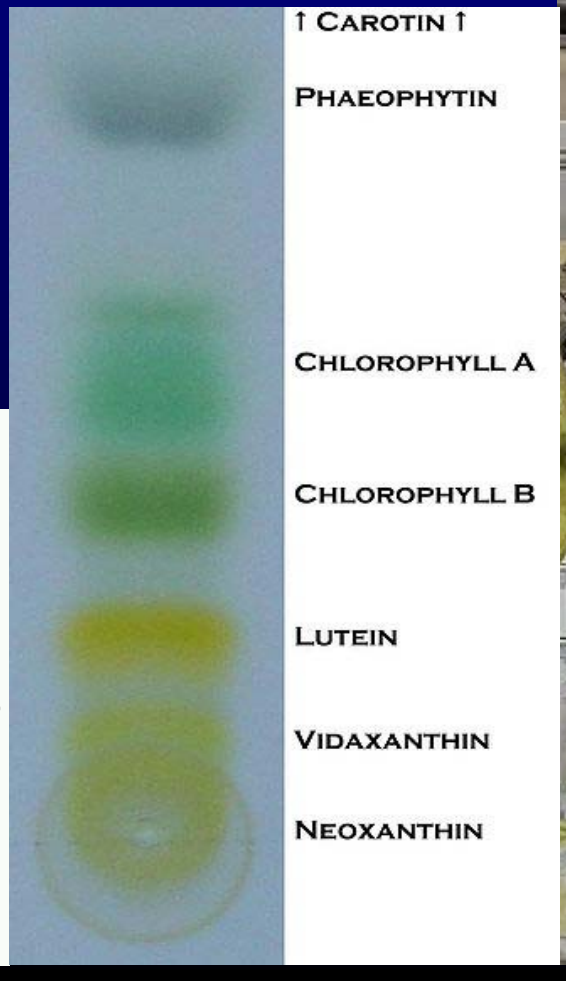
Paper chromatography:
- A paper serves as stationary phase
- Separating and identifying mixtures by colour

Thin layer chromatography:
- Thin layer of silica gel, alumina or cellulose adsorbed on an inert substrate

Column chromatography:
- The stationary phase is packed in a column



From wikipedia.org



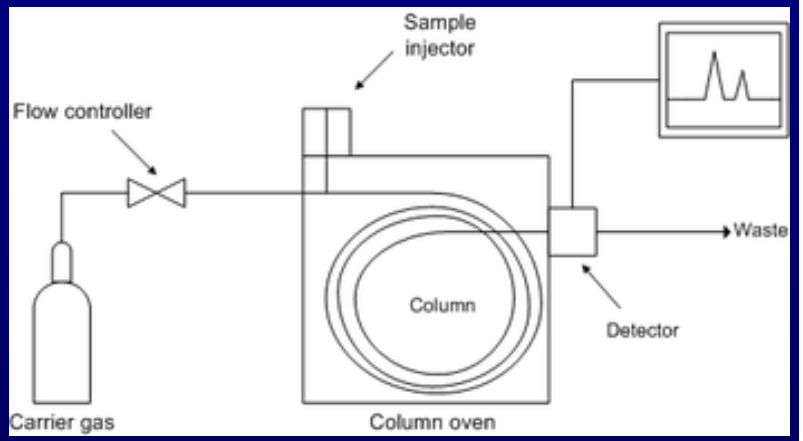
Chromatography: Types

Based on physical state of mobile phase

Gas chromatography: Mobile phase is gas like He

→ Applications: Analytical chemistry, petrochemical, environmental monitoring

→ Not good for bimolecules e.g. protein due to high heat



Liquid chromatography (LC): Mobile phase is liquid

→ In the most simple case, gravity flow is possible

→ High performance liquid chromatography= High pressure liquid chromatography (HPLC) as the modern form, also nicknamed “high price liquid chromatography” because of the expensive machinery required

From our lab

Chromatography: Types

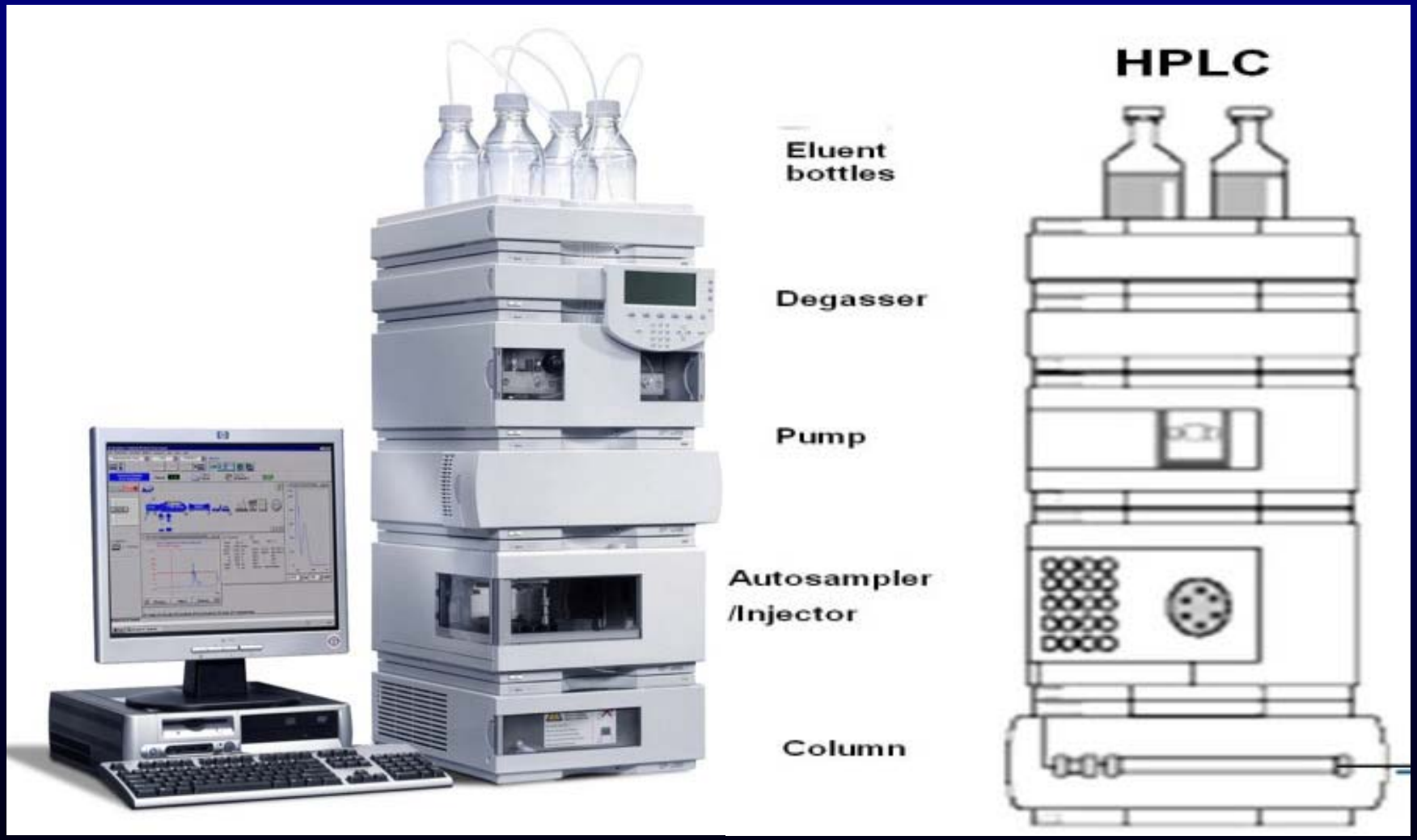
High performance liquid chromatography

Optimized for rapid high resolution separations
-Very high efficiency HPLC columns with inert packing materials
- Fine particle packing (5μm) providing larger surface for interaction
- HPLC high pressure pumps with very constant flow (6000-10000 psi)
-Unique high accuracy, low dispersion, HPLC sample valves
(sub μl - few μl)
- Extremely precise gradient mixers (optional).
- High sensitivity low dispersion HPLC detectors

Applications
quality control, process control, forensic analysis, environmental monitoring and clinical testing

Chromatography: Types

High performance liquid chromatography



Chromatography: Types

High performance liquid chromatography

Normal phase (NP-HPLC):

Polar stationary phase e.g. silica
Non polar mobile phase e.g. Toluene
Polar interaction
Non polar ⟶ Polar

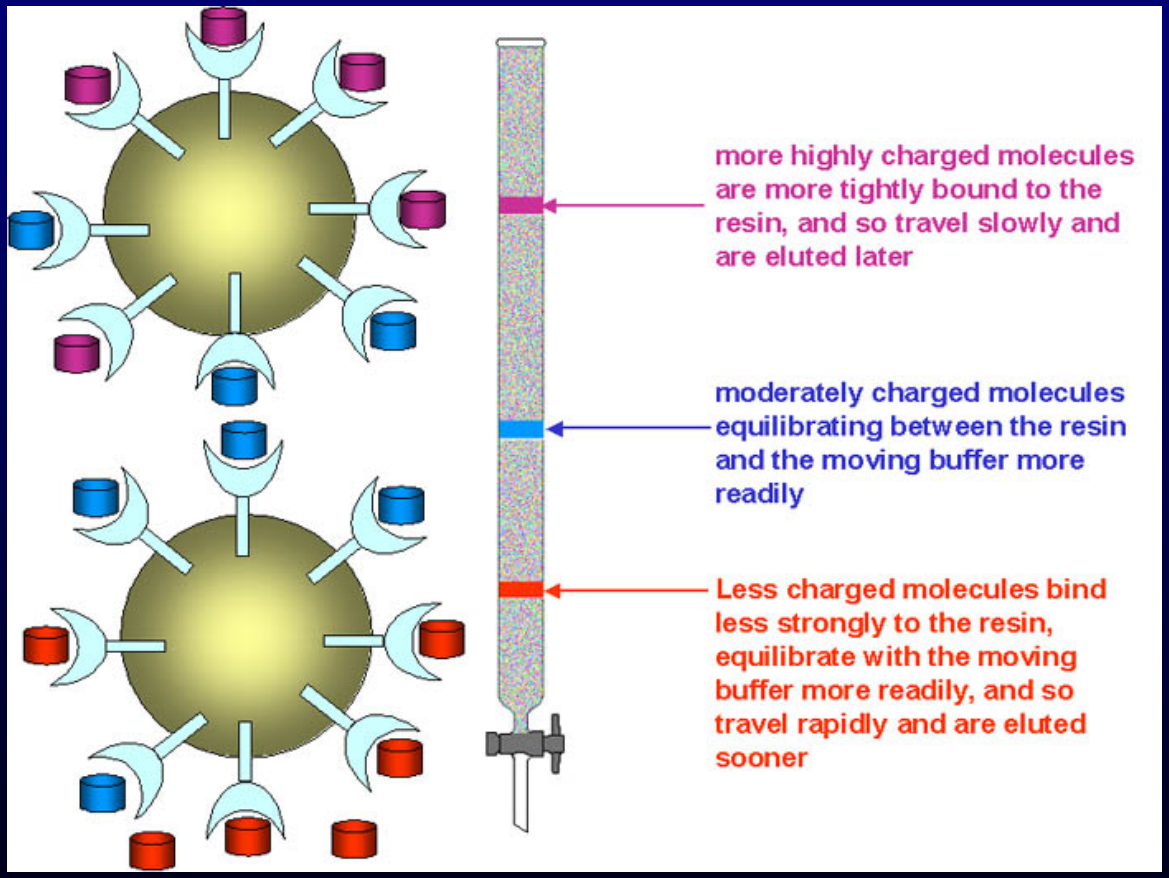
Reverse phase (RP-HPLC):

Non polar stationary phase e.g. C18
Polar mobile phase such as water
Hydrophobic interaction
Retention time is proportional to the contact surface area around the non-polar segment of the analyte
Polar ⟶ Non polar

Chromatography: Types

Ion exchange chromatography

Principle: highly charged proteins bind stronger to the column material, so that they elute at higher salt concentrations in the buffer
than less charged proteins



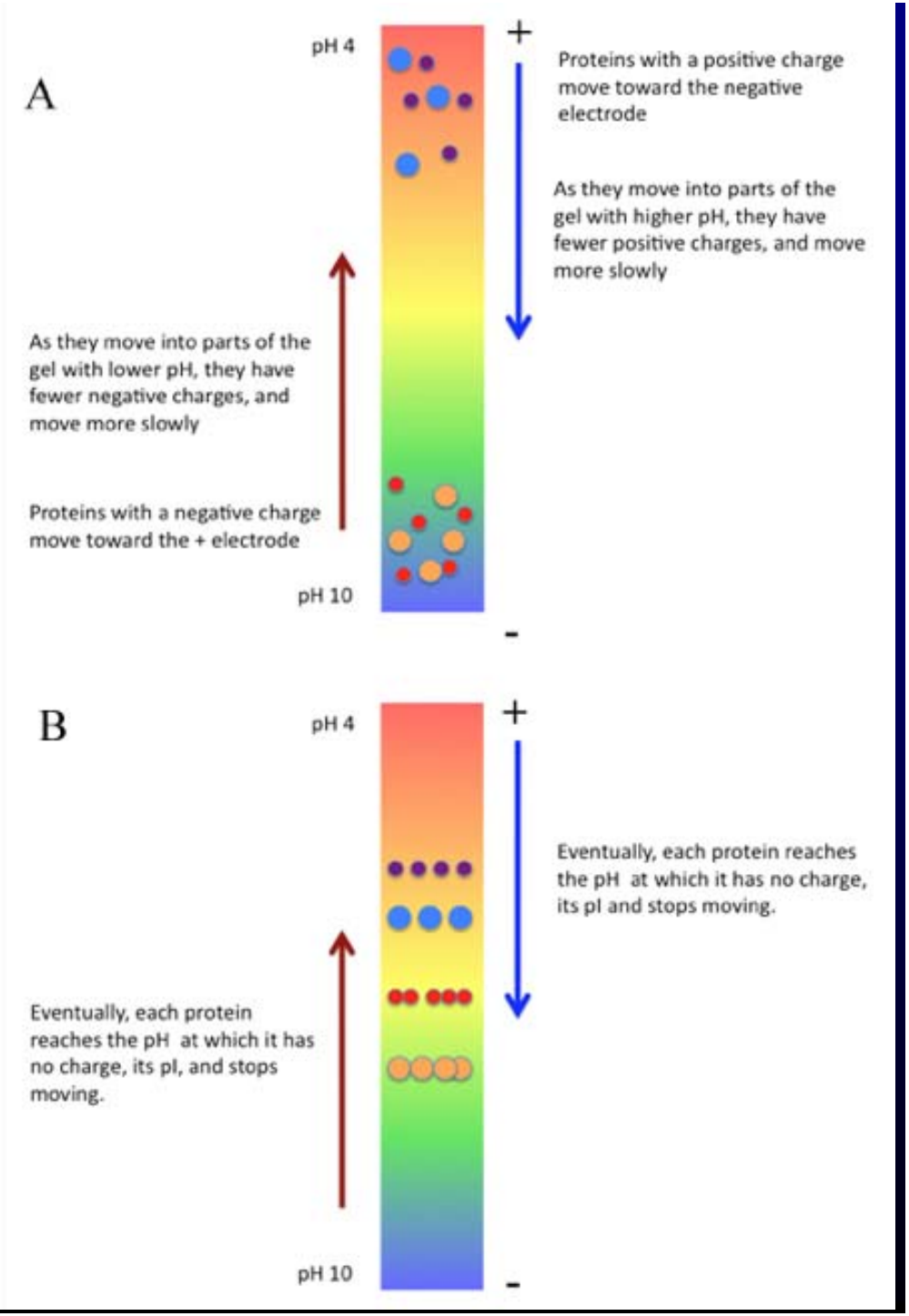
From: www.ucl.ac.uk/~ucbcdab/enzpur/ionX.htm

foto of phycobiliprotein purification in the lab of H. Küpper on a MonoQ anion exchange column

Chromatography: Types

Isoelectric focussing chromatography (IEF-HPLC)

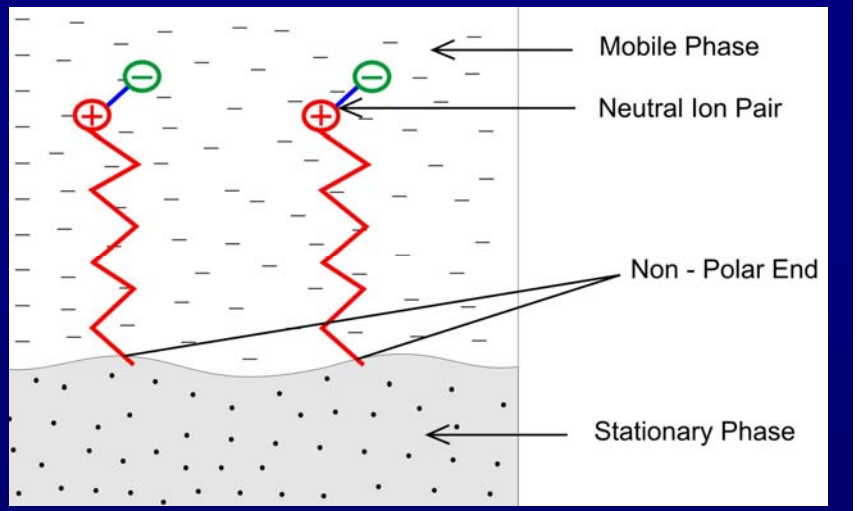
- Isoelectric focussing (IEF) separates proteins by their isoelectric point. The proteins remain where the net charge of the protein is zero, i.e. balance between protonation of carboxyl groups and deprotonation of amino groups is achieved



Chromatography: Types

Ion pair chromatography

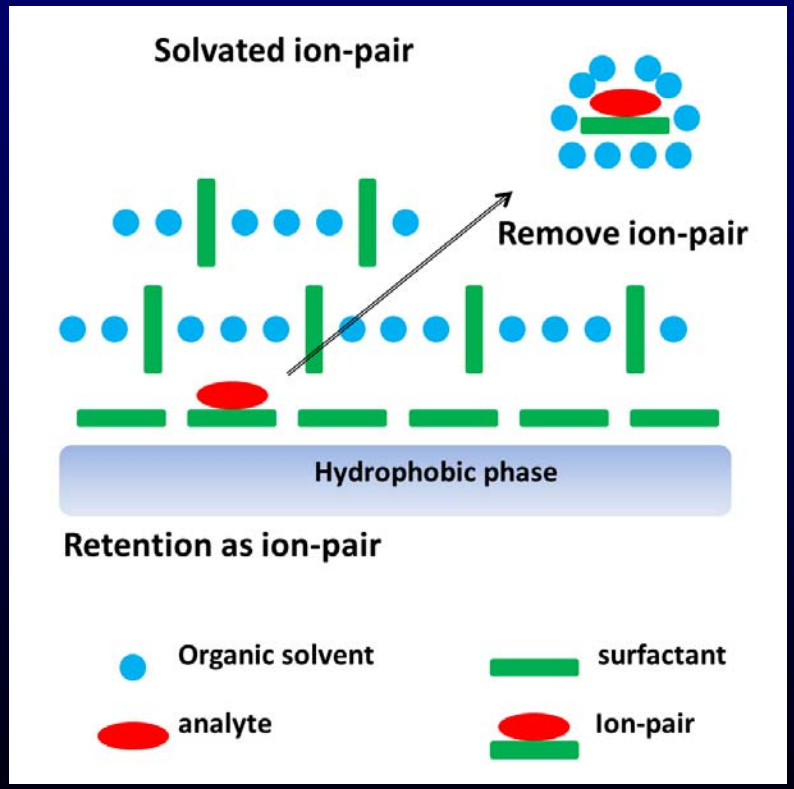
- Ion Pair Chromatography is a method for improving the separation of charged analytes on reversed-phase HPLC columns
-The ion pair reagents comprise of an alkyl chain with an ionizable terminus



From : lab-training.com

Advantages over ion exchange

- Simple preparation of buffers
- Wide choice of carbon chain lengths for improved retention and separation
- Significantly reduced separation time
- Simultaneous separation of both ionized and nonionized solutes
- Highly reproducible results
- Improved peak shape

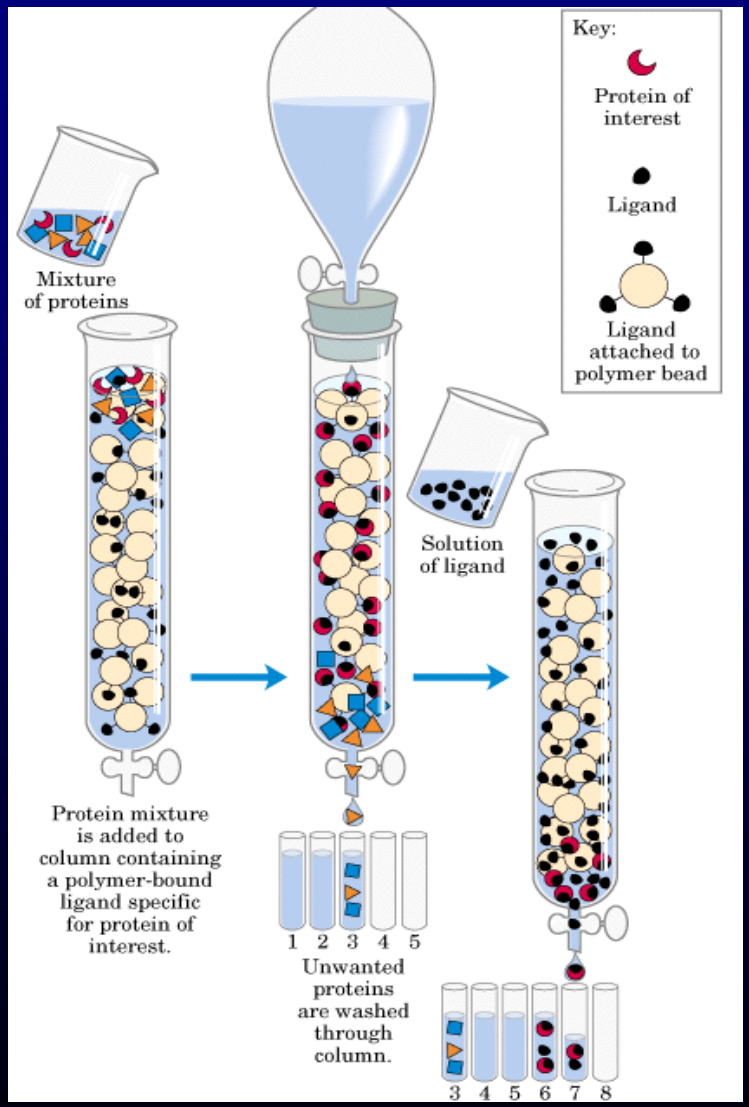


From : atlasofscience.org

Chromatography: Types

Affinity chromatography

Based on a highly specific interaction between analyte and stationary phase



dolly.biochem.arizona.edu/.../methods.html

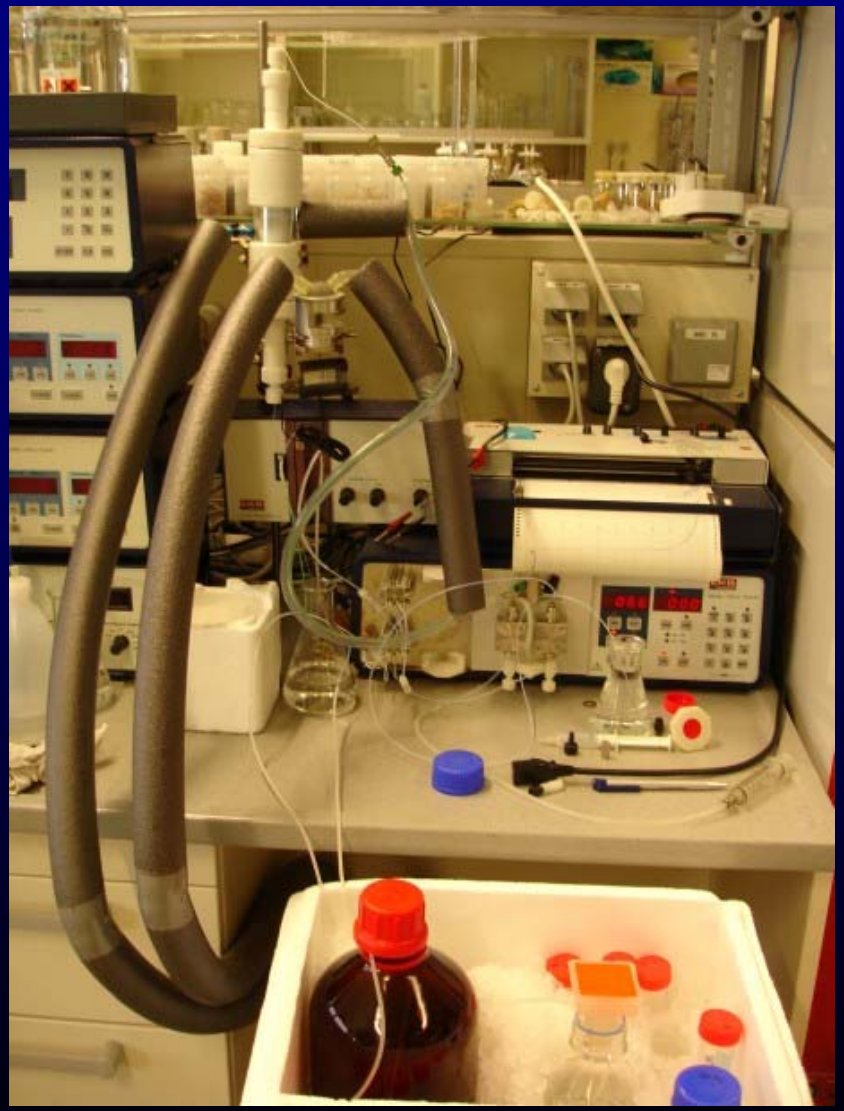


foto of TcHMA4 purification in the lab of H. Küpper on an IMAC column

Chromatography: Types

Hydrophobic interaction chromatography (HIC)

- Solute equilibrates between a solid hydrophobic stationary phase and the eluent
- separation is based on hydrophobicity of the protein surface
- choice of column important – different for membrane vs. soluble proteins!

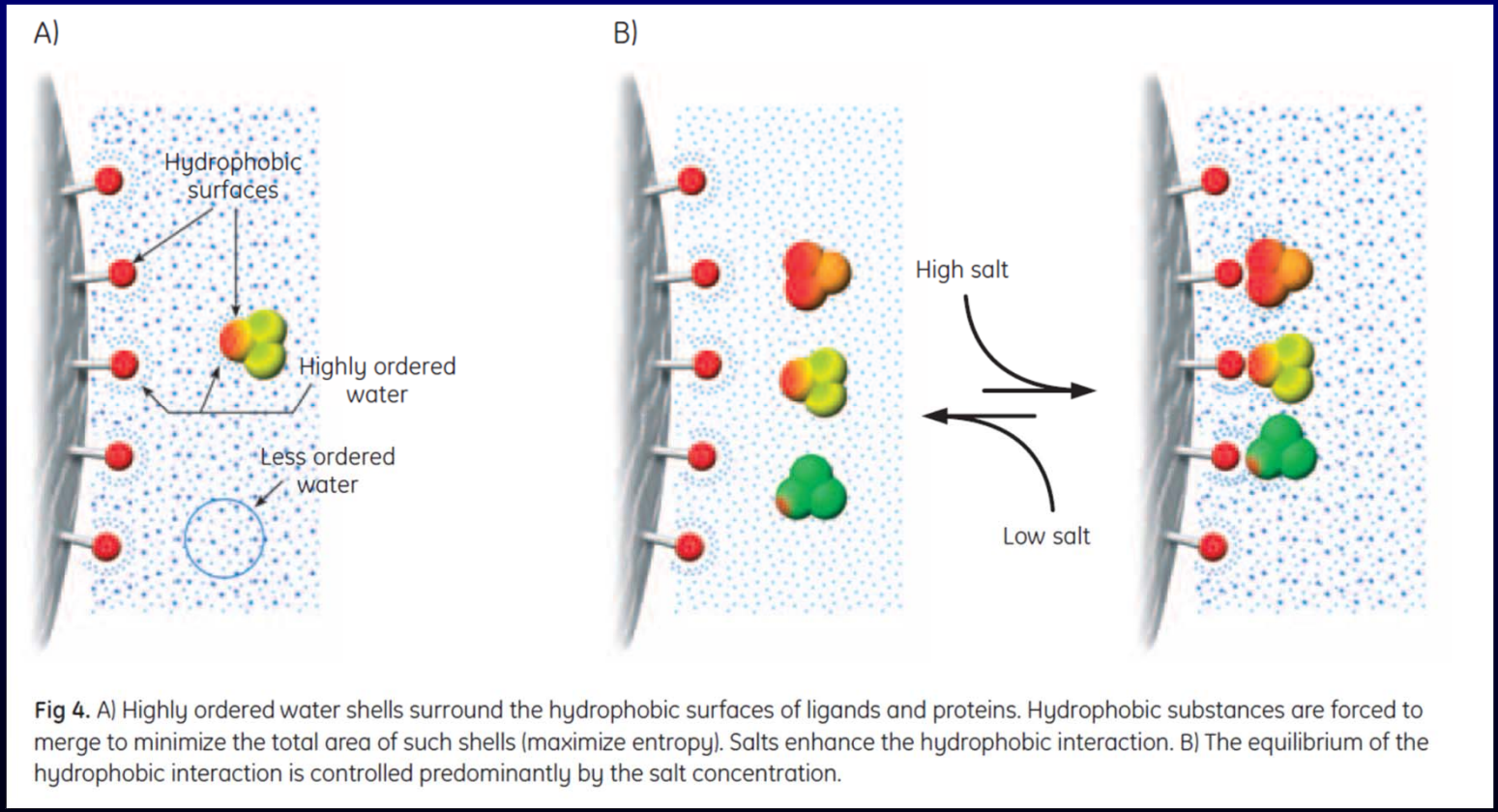


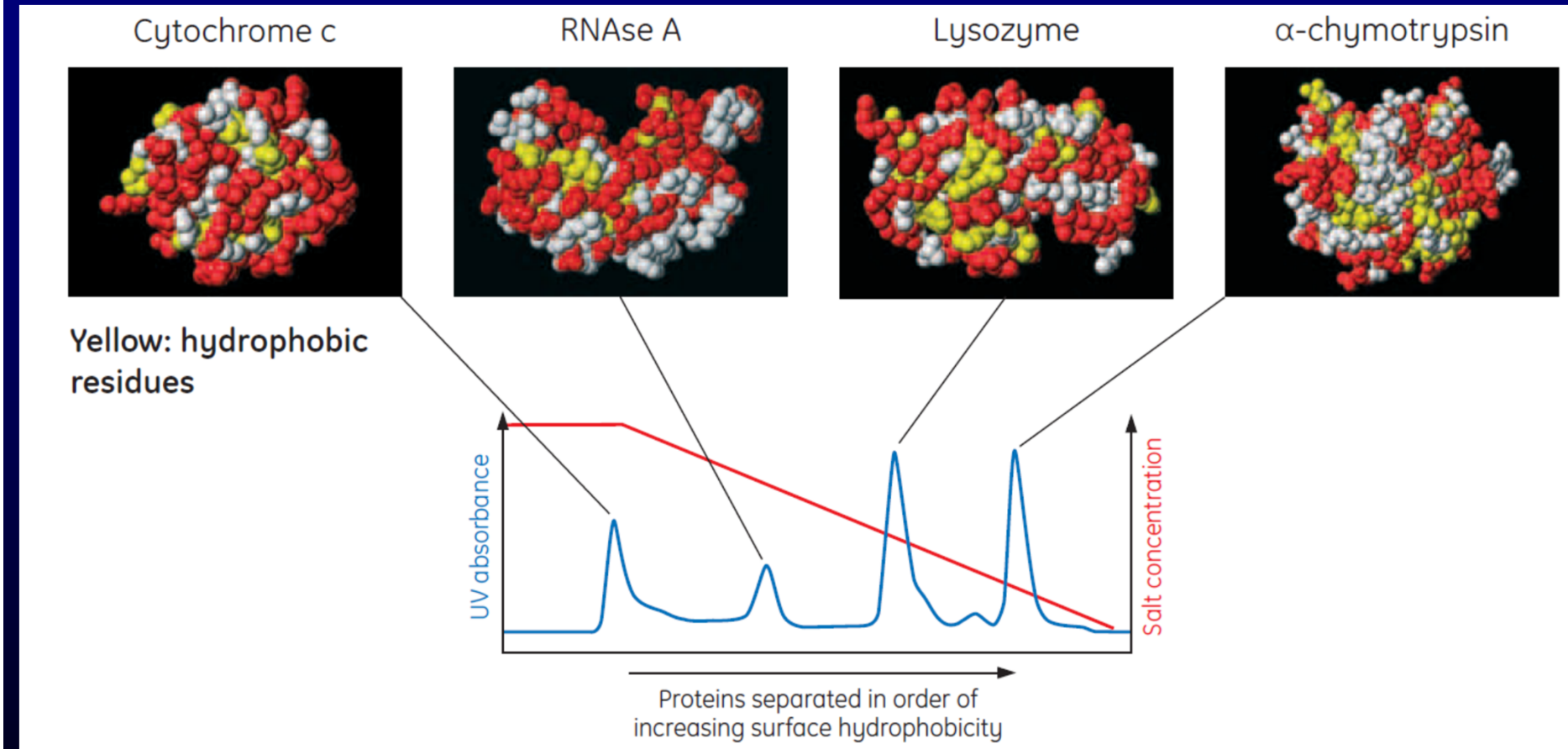
Fig 4. A) Highly ordered water shells surround the hydrophobic surfaces of ligands and proteins. Hydrophobic substances are forced to merge to minimize the total area of such shells (maximize entropy). Salts enhance the hydrophobic interaction. B) The equilibrium of the hydrophobic interaction is controlled predominantly by the salt concentration.

From product information of GE healthcare

Chromatography: Types

Hydrophobic interaction chromatography (HIC)

- separation is based on hydrophobicity of the protein surface: example

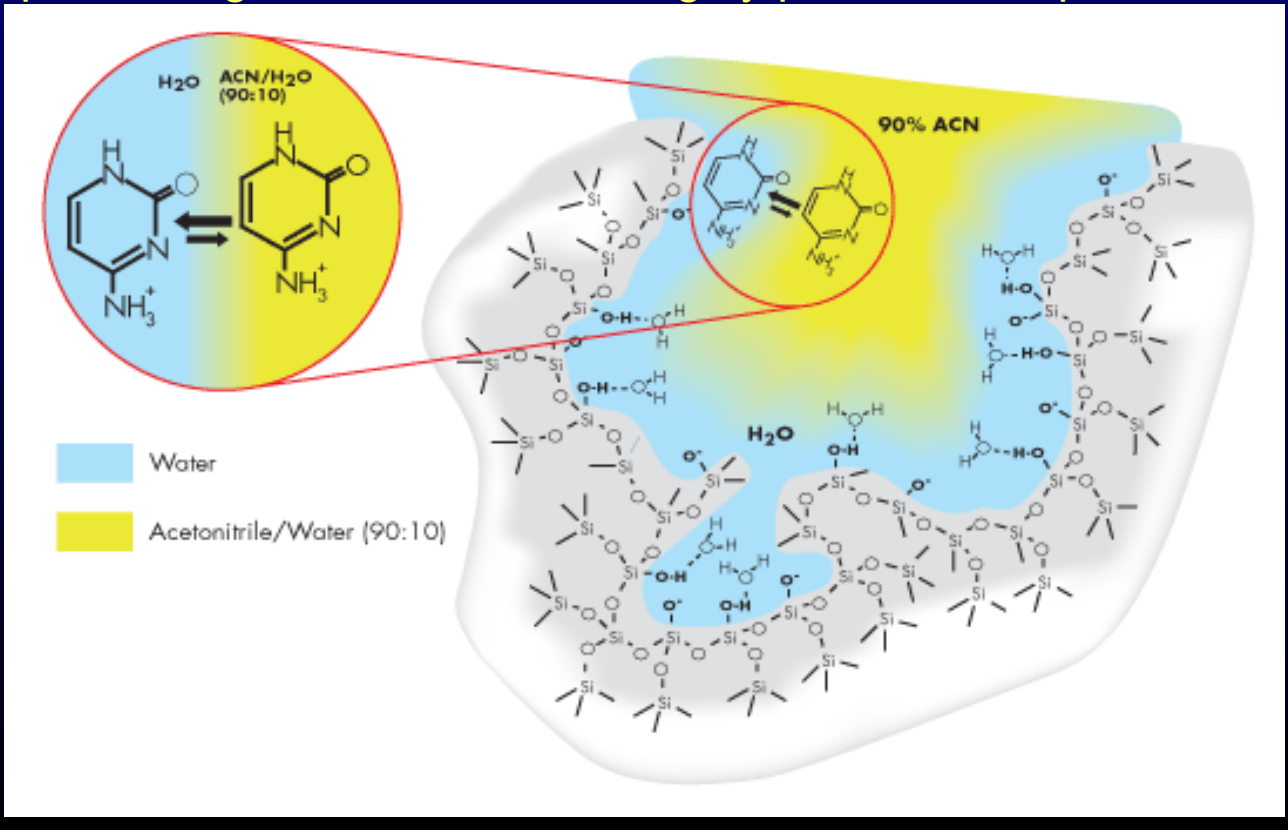


From product information of GE healthcare

Chromatography: Types

Hydrophilic interaction chromatography (HILIC)

-A kind of partition chromatography
- solute equilibrate between a liquid stationary phase and eluent
-Separation based on polar differences
-Can separate acid, base and neutral molecule in single chromatogram
-Good for separation of very polar compounds such as amino acids, glycopeptides, oligonucleotides, and highly polar natural products

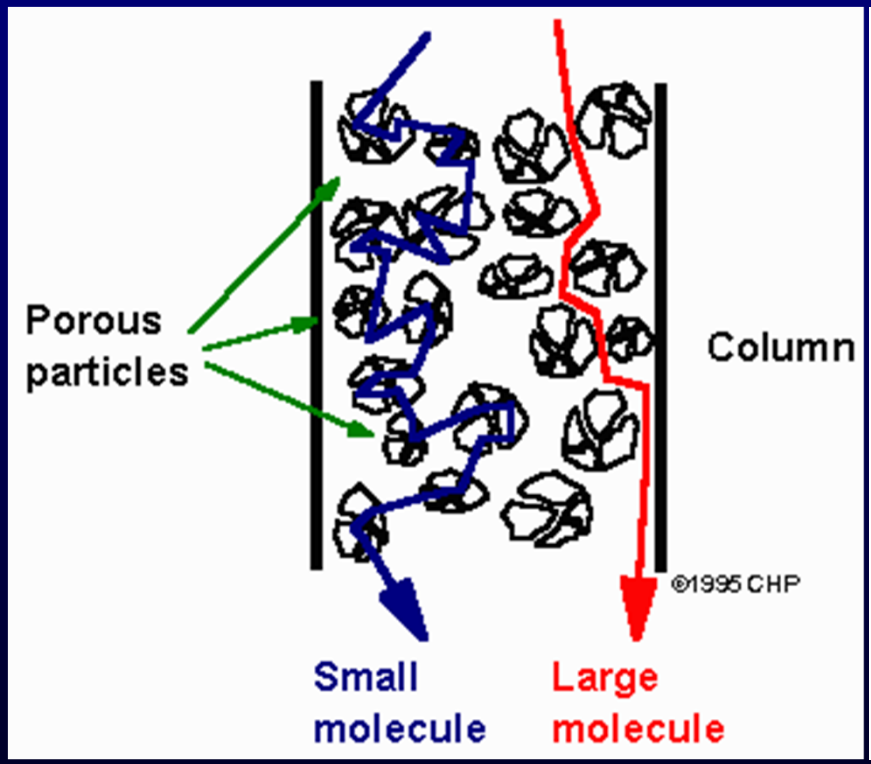


From waters.com

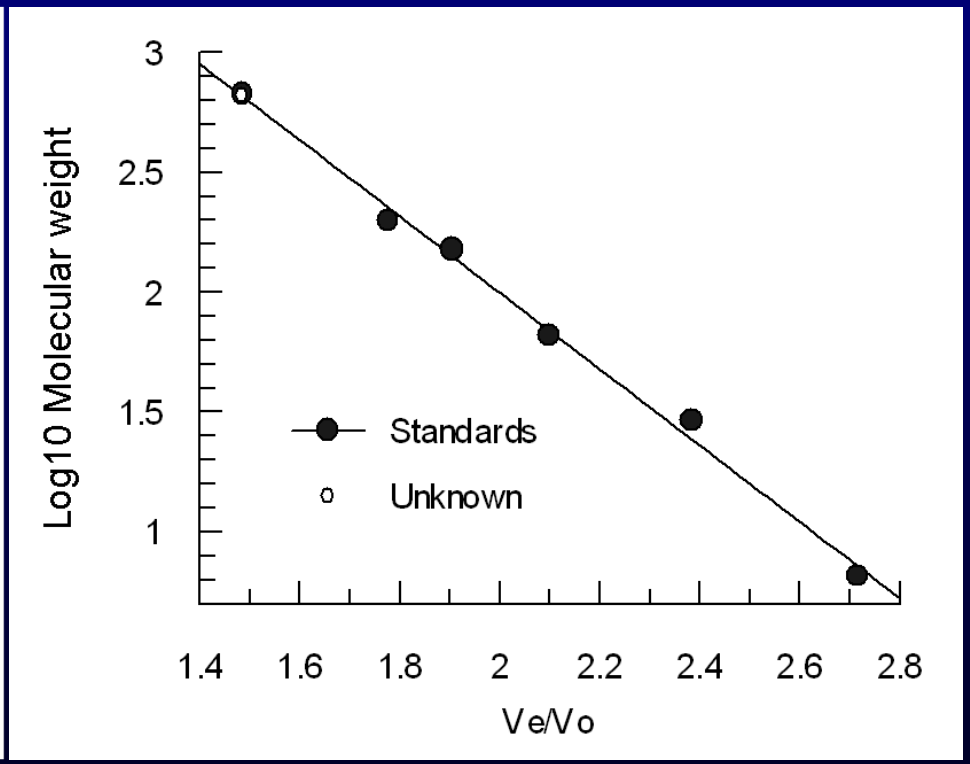
Chromatography: Types

size exclusion chromatography

Principle: Small proteins can enter more of the pores in the column material than large proteins, so that small proteins migrate **slower**



From: elchem.kaist.ac.kr



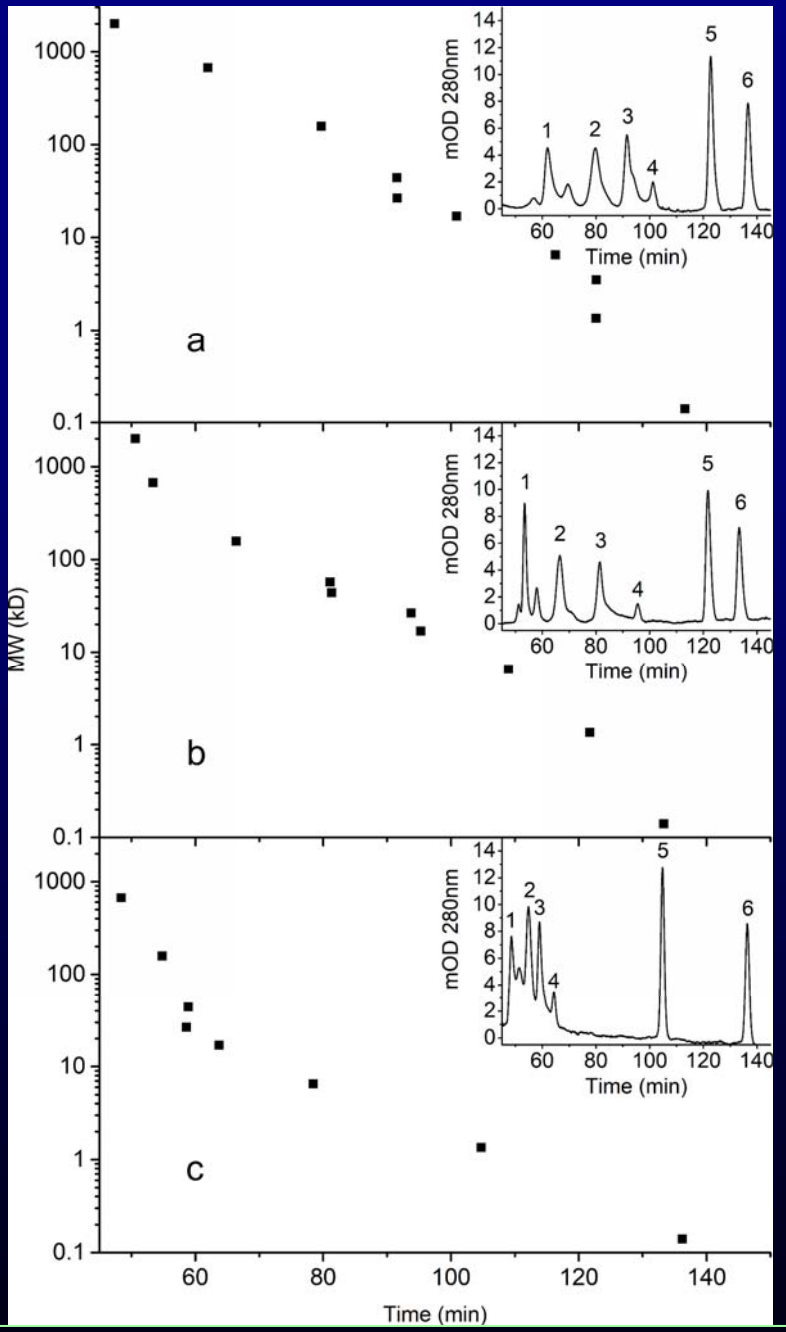
From: http://en.wikipedia.org/wiki

Chromatography: Types

size exclusion chromatography

Optimisation of column choice for different protein sizes.

(a) widest MW range with increased MW resolution in the centre: 1x Superose 6 increase, 1x Superose 12, 1x Superdex200 increase.
(b) focus on medium to high MW resolution: 1x Superdex75 increase, 2x Superdex200 increase.
(c) focus on ultra-low MW range but medium MW range covered: 2x Superdex 30 increase, 1x Superose 12.



Küpper H, Hussain Bokhari SN, Jaime Perez N, Lyubenova L, Ashraf N, Andresen E (2019) Analytical Chemistry 91, 1710961-10969

Chromatography: Types

"Special" technique?

Fast protein liquid chromatography (FPLC)

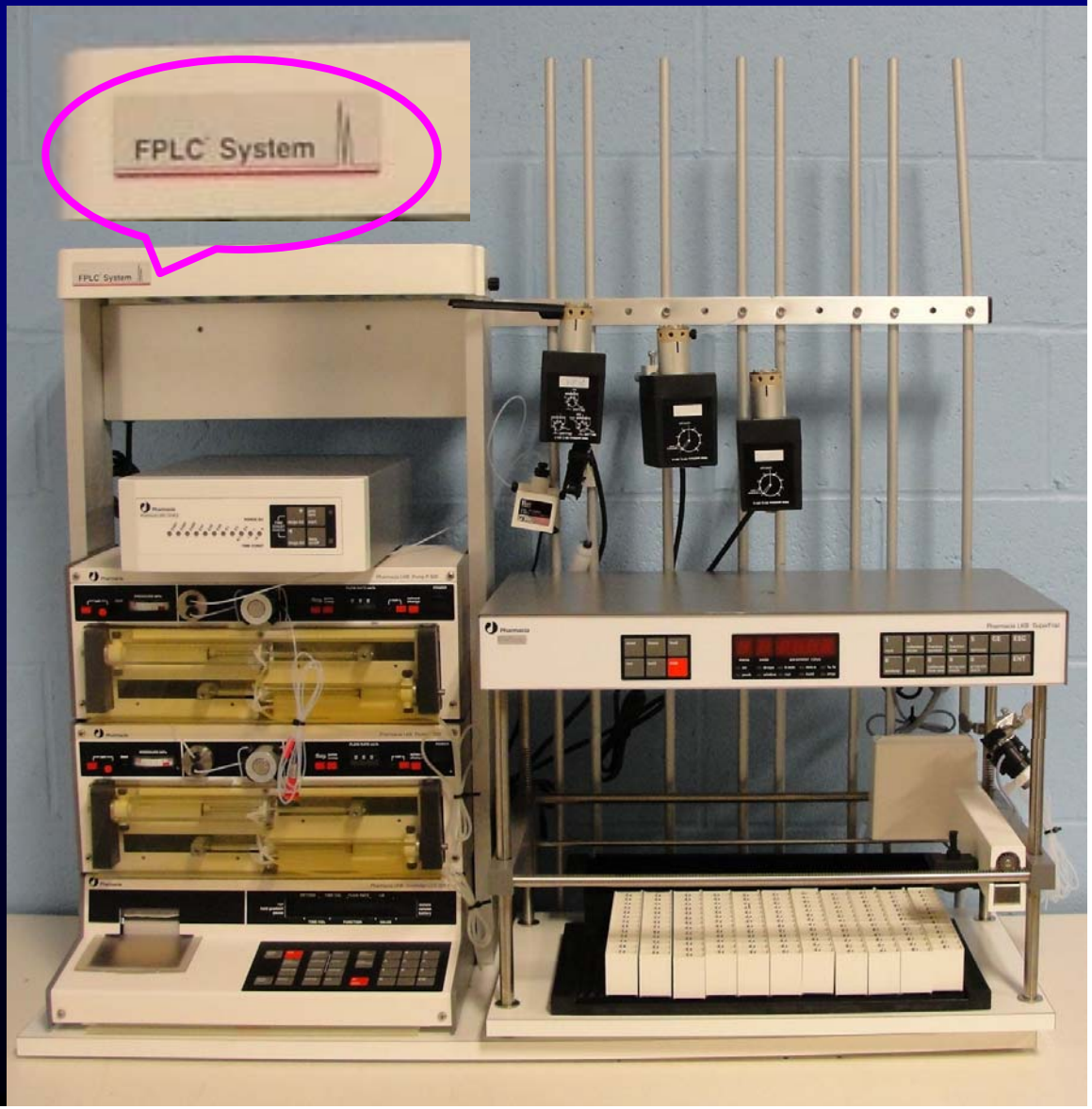
-Also called as fast performance liquid chromatography, a variant of HPLC

- Now often, wrongly, used as a synonym for HPLC of proteins, BUT: Originally a **trademark** of LKB→Pharmacia→Amersham→ GE healthcare→Cytiva

- Was designed by LKB/Pharmacia for protein purification in 1982

- Original FPLC operates at low pressure typically less than 5 bar, modern protein chromatography often up to 100 bar, i.e. typical HPLC range

- flow rate is relatively high, typically 1-5 ml/min.



Hyphenated techniques

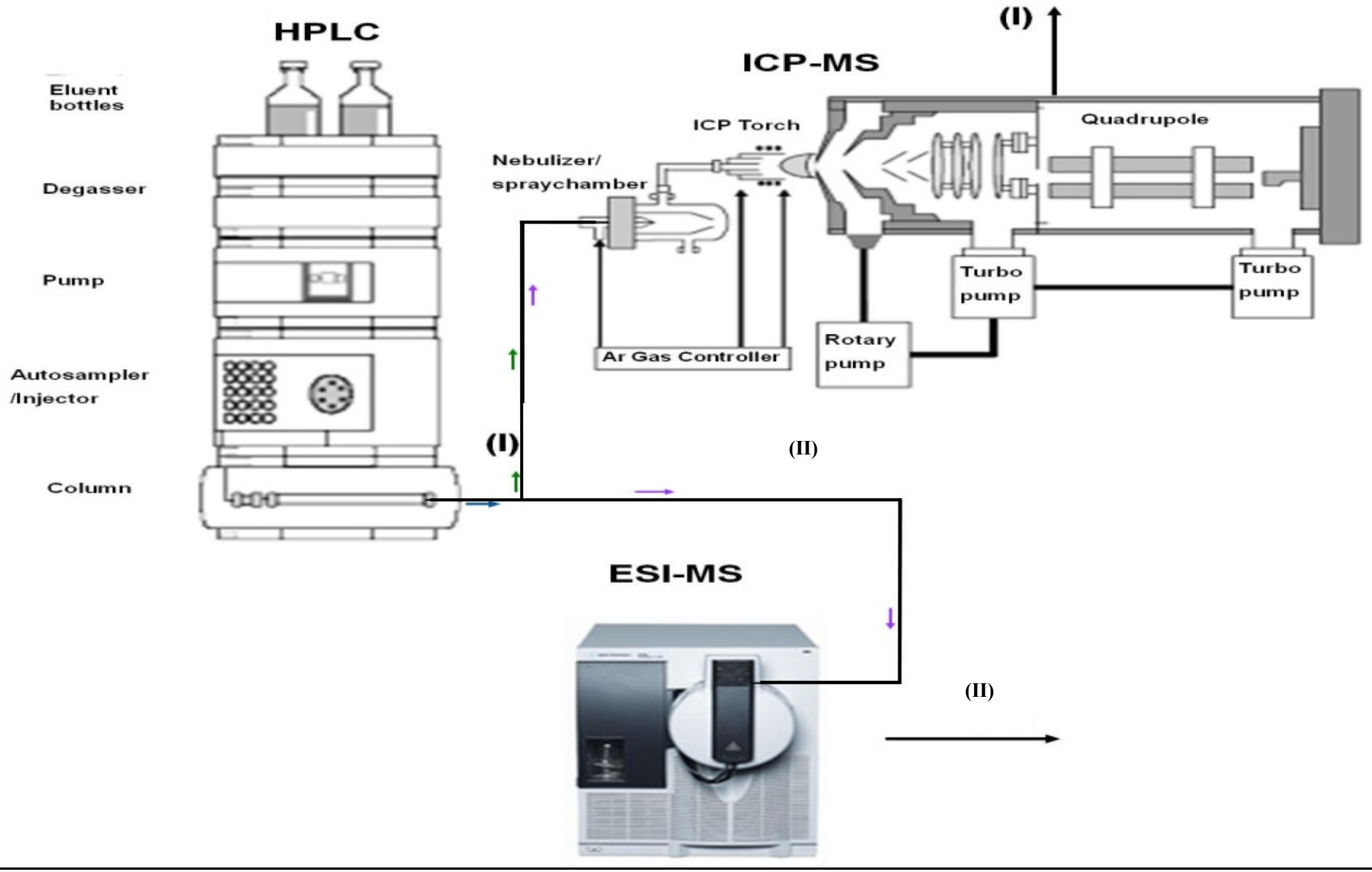
Hyphenated Techniques combine chromatographic and spectral methods to exploit the advantages of both.

Chromatography - Produces pure or nearly pure fractions of chemical components in a mixture.

Spectroscopy – Produces selective information for identification using standards or library spectra.

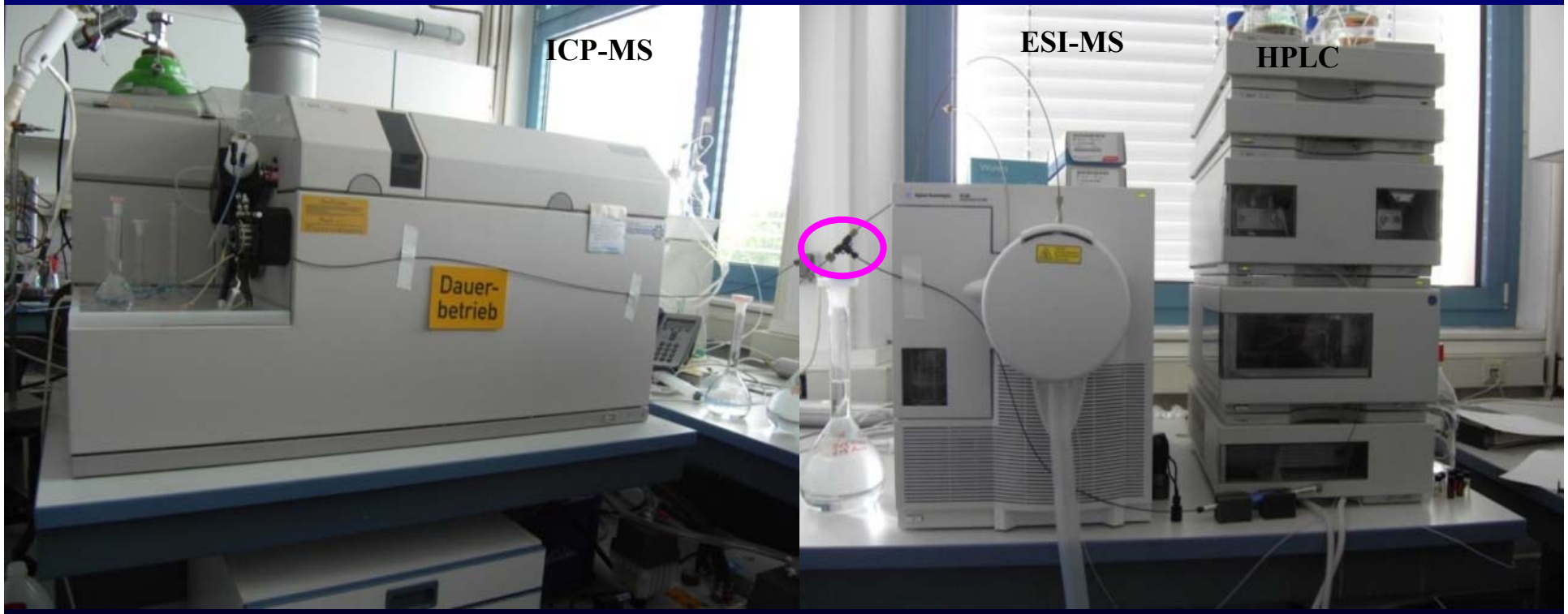
Hyphenated techniques

HPLC-ICP-MS-ESI-MS



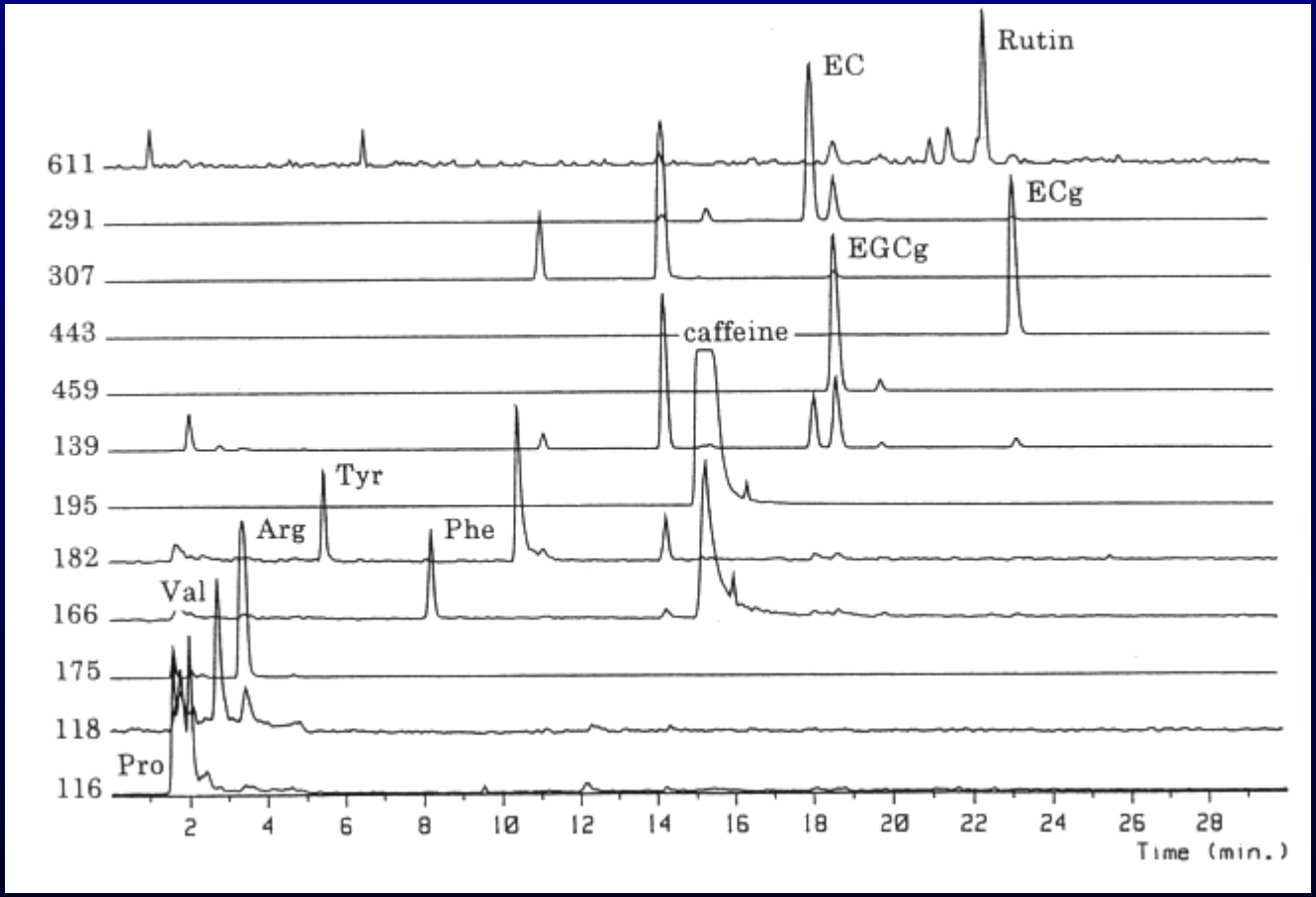
Hyphenated techniques

HPLC-ESI-MS-ICP-MS



Hyphenated techniques

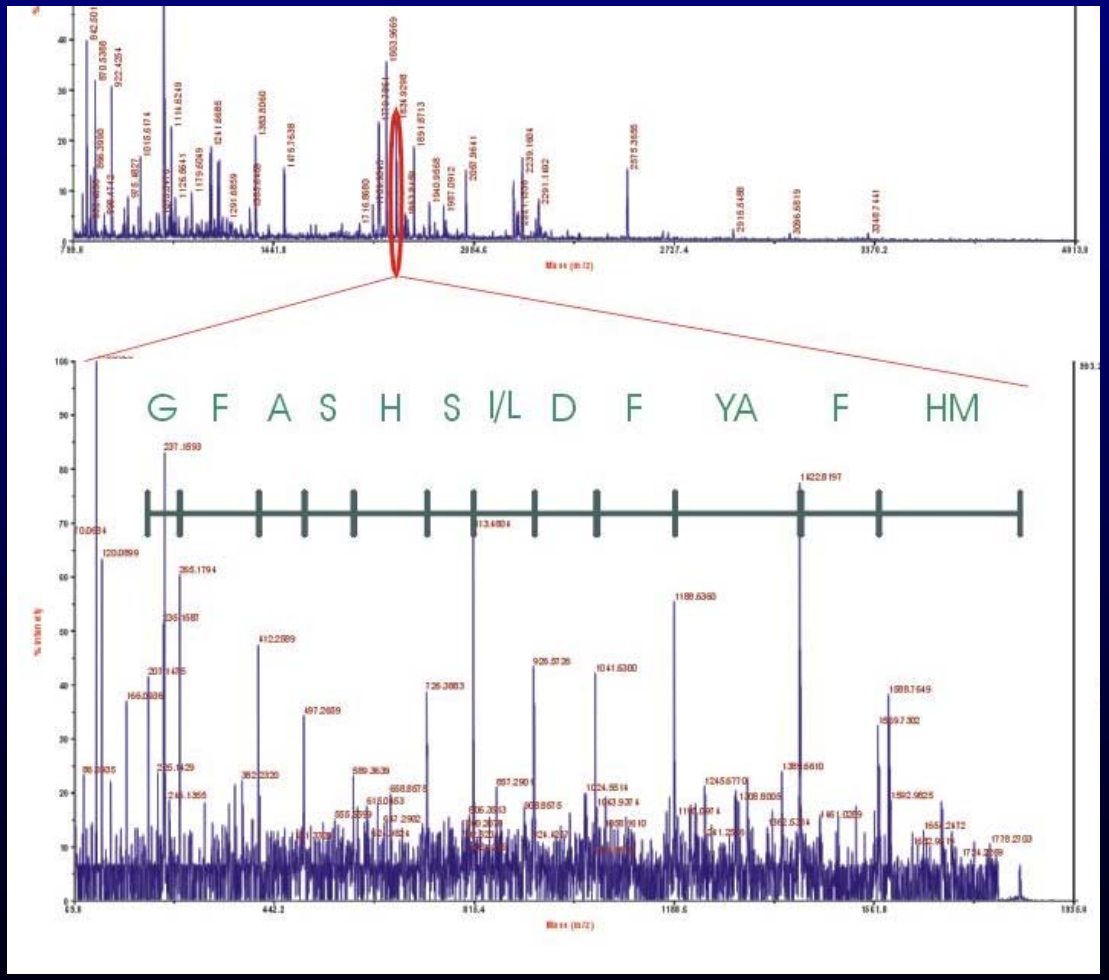
LC-MS Data



JEOL HIGHTECH LTD http://www.datum.jeol.co.jp/hightech/ai-msdata-3.html

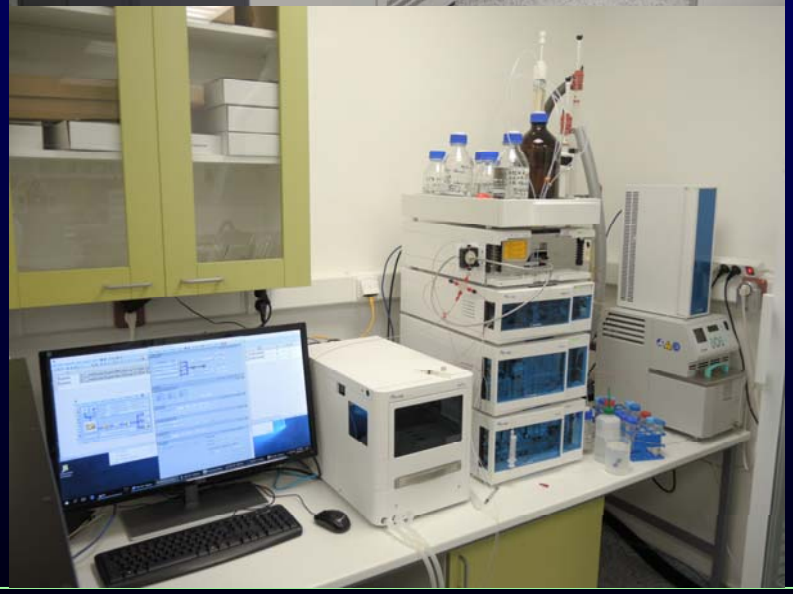
Hyphenated techniques

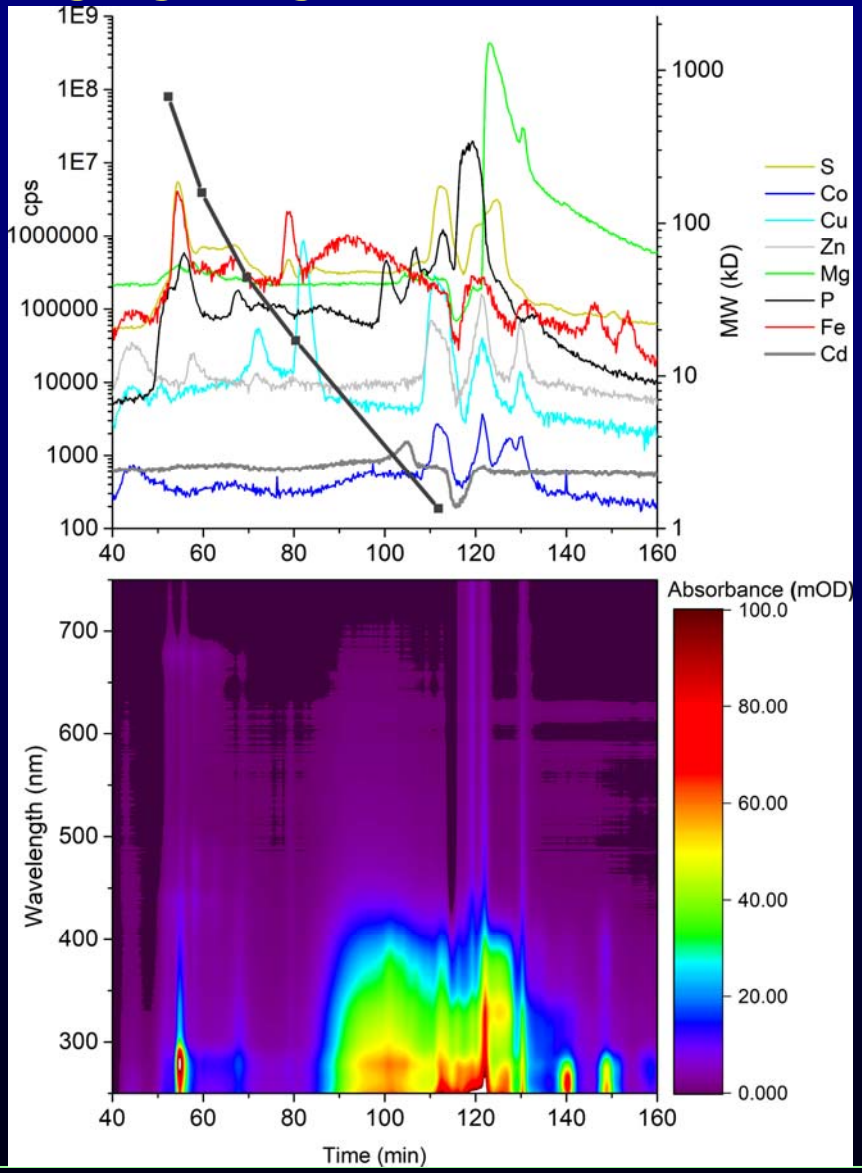
HPLC-ESI-MS-MS



Hyphenated techniques

HPLC-UVVIS-ICPMS





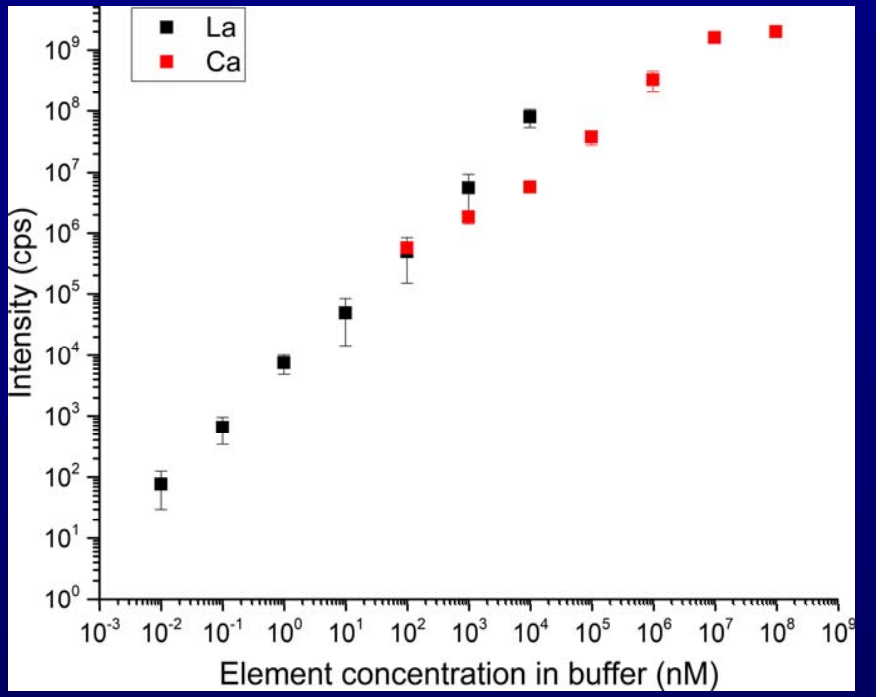
Left: Photo from our lab; *right:* Küpper H, Hussain Bokhari SN, Jaime Perez N, Lyubenova L, Ashraf N, Andresen E (2019) Analytical Chemistry 91, 1710961-10969

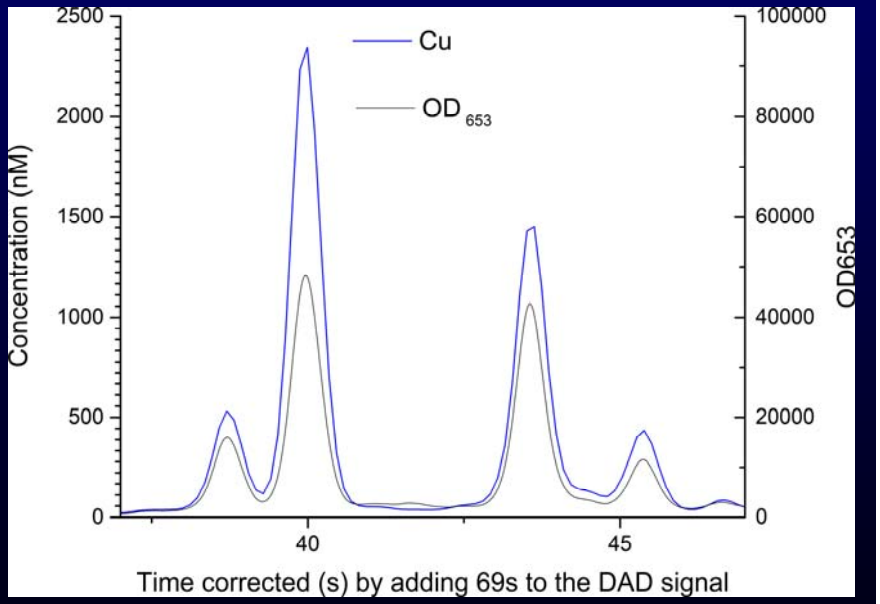
Hyphenated techniques

HPLC-UVVIS-ICPMS

Necessary calibrations for quantitative work

(a) Test of linearity of the system in the required concentration range
(b) Adjustment of timing to prevent problems of signal delay mismatch between the systems





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All slides of my lectures can be downloaded from my workgroup homepage

Biology Centre CAS → Institute of Plant Molecular Biology → Departments → Department of Plant Biophysics and Biochemistry,

or directly

http://webserver.umbr.cas.cz/~kupper/AG_Kuepper_Homepage.html